

A pyrrole-imidazole polyamide is active against enzalutamide-resistant prostate cancer

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Running Title: **Polyamide efficacy in enzalutamide resistant prostate cancer**

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Conflict of Interest: P.B.D., F.Y., N.G.N. founded Gene Sciences, Inc. which is developing therapeutics for enzalutamide-resistant prostate cancer.

Abstract

The LREX' prostate cancer model is resistant to the antiandrogen enzalutamide via activation of an alternative nuclear hormone receptor (NHR), glucocorticoid receptor (GR), which has similar DNA binding specificity to the androgen receptor (AR). Small molecules that target DNA to interfere with protein-DNA interactions may retain activity against enzalutamide-resistant prostate cancers where ligand binding domain antagonists are ineffective. We reported previously that a pyrrole-imidazole (Py-Im) polyamide designed to bind the consensus androgen response element half-site has antitumor activity against hormone-sensitive prostate cancer. In enzalutamide-resistant LREX' cells, Py-Im polyamide interfered with both androgen receptor- and glucocorticoid receptor-driven gene expression, while enzalutamide interfered with only that of androgen receptor. Genomic analyses indicated immediate interference with the androgen receptor transcriptional pathway. Long-term treatment with Py-Im polyamide demonstrated a global decrease in RNA levels consistent with inhibition of transcription. The polyamide was active against two enzalutamide-resistant xenografts with minimal toxicity. Overall, our results identify Py-Im polyamide as a promising therapeutic strategy in enzalutamide-resistant prostate cancer.

Introduction

Prostate cancer (CaP) is the second leading cause of cancer death in American men with 26,000 deaths annually (1), the majority from metastatic, castrate resistant CaP (mCRPC), in which androgen deprivation therapy (ADT), which suppresses AR signaling, is ineffective. Enzalutamide, a potent AR-ligand binding domain (LBD) antagonist, is effective against mCRPC and is a current standard of care (2). Unfortunately, *de novo* or acquired resistance to enzalutamide is common (3); overcoming this is an unmet need.

Mechanisms of enzalutamide resistance include restoration of AR signaling through LBD mutations or expression of transcriptionally active splice variants lacking the LBD (4), bypass of AR signaling through alternative NHRs (5), or development of complete independence from AR signaling (6). GR is a NHR with a sequence preference similar to AR (7). After enzalutamide treatment, the LREX' cell line highly expresses GR, which drives enzalutamide resistance by regulating gene expression significantly overlapping that of AR, suggesting CaPs co-opt GR to progress through AR antagonism (5). Furthermore, GR expression in mCRPC associates with poor response to enzalutamide (5). Therefore, interference with the NHR-DNA interface may overcome enzalutamide resistance.

A Py-Im polyamide (**ARE-1**) is effective against hormone sensitive LNCaP xenografts with minimal host toxicity (8). Py-Im polyamides are minor groove DNA binding small molecules with modular sequence specificity and high affinity (9). Polyamide-DNA binding induces widening of the minor groove and compression of the opposing major groove (10), interfering with transcription factor-DNA interactions and the transcriptional machinery (11,12). A polyamide targeted to the ARE might prevent AR and GR signaling, and transcription.

We hypothesized that **ARE-1** may be effective against enzalutamide-resistant CaP. We report **ARE-1** efficacy against enzalutamide resistant VCaP and LREX' CaP models in cell culture and xenografts. Mechanistic studies reveal immediate interference with androgen induced gene expression and reduced transcription after long-term treatment.

Materials and Methods

Cell culture conditions and cytotoxicity assays. The LREX' and LNCaP/AR cell lines were gifts from Charles Sawyers (Memorial Sloan Kettering) and received 2014 and 2007, respectively. The VCaP cell line

was a gift from Kenneth Pienta (University of Michigan Medical School) and received 2012. Cells were maintained as previously described (5,8,11–13), and were used within 10 passages from thawing. Cells were validated to parental cell lines by STR profile at IDEXXX Bioresearch following experimentation and confirmed to be *Mycoplasma* free. WST-1 assay (Roche) was used to measure cytotoxicity. Long-term toxicity in VCaP cells was assayed by cell counting.

Confocal imaging. Imaging was as described (8). Briefly, 2 μ M of **ARE-1-FITC** was added for 16hours, washed with PBS, and imaged on a Zeiss LSM 5 Exciter.

Gene expression analysis. LNCaP/AR and LREX' cells were cultured for 72hours after plating in phenol-red free RPMI1640 (10% CT-FBS) in six well plates at 40,000 and 50,000cells/mL, respectively. LNCaP/AR cells were treated with 10 μ M **ARE-1**, bicalutamide (bic), or enzalutamide (enz, Aurum Pharmatech) for an additional 48, 2, and 2hours, respectively, prior to treatment with 1nM DHT or ethanol for 16hours. LREX' cells were treated with 10 μ M **ARE-1** for 16hours prior to induction with 1nM DHT or 100nM dexamethasone (dex) for 8hours. RNA extraction (RNEasy columns, Qiagen), cDNA generation (Transcriptor First Strand cDNA kit, Roche), and qRT-PCR (SYBR Green Master Mix, Applied Biosystems, ABI7300 instrument) were as described (8,11,12). Expression was normalized to β -glucuronidase.

RNAseq analysis. LREX' cells were plated at 50,000cells/mL in 10cm² dishes, treated with or without 10 μ M of **ARE-1** in fresh media, incubated 16hours, and induced with 1nM DHT for 8hours. Tumor samples were homogenized mechanically. Total RNA was triazol extracted, sequenced (Illumina HiSeq2000), mapped against the human genome (hg19) with Tophat2 using Ensembl GRCh37 gene annotations. Human and mouse reads from tumor samples were parsed with BBSplit and unique reads were mapped. Htseq-count was used for exon alignment and DESeq2 for differential expression. Gene set enrichment analysis (GSEA) was performed on genes with padj<0.05 and fold change \geq 1.6 for cell samples and padj<0.05 for tumor samples.

Nascent RNA measurement. LREX' cells were plated at 100,000cells/mL in 96 well plates in RPMI1640 (20% FBS and 1 μ M enz), adhered for 24hours, dosed with **ARE-1**, incubated for 48hours. The Click-iT® RNA Alexa Fluor® 488 HCS kit was used for dye conjugation and incorporation of 5-ethynyl uridine (5-EU) was measured on a Flexstation 3 plate reader.

Flow cytometry. LREX' cells were plated at 100,000cells/mL in 175cm² flasks, adhered 24hours, incubated with 10μM **ARE-1** 24, 48, and 72hours, then with 300μM 5-EU in fresh media. Cells were detached by Accumax or Accutase, and Alexa Fluor® 488 azide dye was conjugated. Cells were passed through 35μm mesh prior to flow, sorted on a FACSCalibur instrument (Beckman-Dickinson), analyzed using FlowJo.

Animal experiments. Animal experiments were performed at Caltech under IACUC approval. VCaP and LREX' cells were engrafted as 1:1 mixtures of 3x10⁶cells in Matrigel (BD Biosciences) into flanks of intact and castrated male SCID mice (Charles River), respectively. LREX' engrafted mice received 10mg/kg enz (oral gavage) daily. Once tumors were 100mm³ (0.5*I*w*w), **ARE-1** was administered subcutaneously to opposing flanks in 20% DMSO:saline. For circulation studies, four C57BL6/J animals were injected subcutaneously with **ARE-1** at 30mg/kg and blood collected retroorbitally. Plasma concentrations of **ARE-1** were analyzed by HPLC, area under the curve (AUC) approximated by the linear trapezoidal method, as described (8).

Immunohistochemistry. Tumors were fixed in neutral-buffered formalin, paraffinized, sectioned, stained as described (12). Quantification of five random fields per slice was performed by ImmunoRatio.

Statistical analysis. Cell culture experiments represent ≥3 independent biological replicates. Sequencing analyses were duplicates for cell culture and quadruplicates for tumor samples. For xenografts, animals were randomly assigned to groups. For circulation experiments, concentrations of **ARE-1** were duplicate measurements. Measurements in cell culture, animal, and immunohistochemistry experiments were assessed by Student's t-test.

Results

ARE-1 is more potent than enzalutamide against CaP cell growth and is not rescued by GR activation

ARE-1 (Fig. 1A) targets the sequence 5'-WGWWCW-3' (W=A or T), similar to the consensus half site recognized by either AR or GR. Nuclear uptake in LNCaP/AR, LREX', and VCaP cells was evaluated using fluorescent analog **ARE-1-FITC** (Supplementary Fig. S1). The LNCaP/AR cell line overexpresses full length AR, modeling castration resistance (14). **ARE-1** reduced proliferation of LNCaP/AR cells more than bicalutamide (Fig. 1B). The VCaP cell line overexpresses AR with modest GR expression, the activation of which reduces the antiproliferative effects of enzalutamide (5). **ARE-1** reduced proliferation of both VCaP and

LREX' cells regardless of induction of AR signaling by 1nM DHT, induction of GR signaling by 100nM dex, or both (Fig. 1C-D). Long-term cell viability studies in VCaP cells show **ARE-1** is more potent than enzalutamide and insensitive to GR activation (Fig. 1D).

Py-Im polyamide attenuates androgen and glucocorticoid driven gene expression

In androgen-depleted conditions, bicalutamide activates AR in the LNCaP/AR cell line (14). Enzalutamide and **ARE-1** demonstrate no agonist activity; **ARE-1** reduced baseline expression of *KLK3* (Fig. 2A). In LREX' cells, **ARE-1** represses *KLK3* and *HOMER2* expression, which are co-regulated by AR and GR (Fig. 2B). While enzalutamide was more potent than **ARE-1** in reducing DHT induced transcription, the opposite was observed with dex induction. Furthermore, co-administration of enzalutamide and **ARE-1** was additive, suggesting **ARE-1** may potentiate enzalutamide's activity.

Global transcriptomic effects of Py-Im polyamides

We performed RNA-seq analysis on three treatment conditions in LREX' cells: vehicle, DHT treatment, and co-treatment with **ARE-1** and DHT, and two conditions in parental LNCaP cells: vehicle, and **ARE-1** treatment. GSEA of affected genes in LREX' cells using the hallmark pathways in the Molecular Signatures Database revealed DHT treatment enriched for the AR signaling pathway as expected (Fig. 2C, Supplementary Fig. S2 and Table S1). DHT-induced LREX' cells treated with **ARE-1** negatively enriched for the AR signaling pathway (NES -3.875) (Fig. 2C, Supplementary Table S1), consistent with interference in AR-driven gene expression by **ARE-1**. Additionally, **ARE-1** treatment negatively enriched for the UV DNA damage response pathway down (NES -4.310) (Fig. 2C). Similarly, **ARE-1** treatment in LNCaP cells negatively enriched for the AR signaling pathway (NES -2.778) and the UV DNA damage response pathway down (NES -2.240) (Fig. 2D, Supplementary Table S1). UV radiation induces DNA helical distortions through formation of pyrimidine dimers and 6-4 photoproducts, which arrest RNA Polymerase II (RNAP2) during elongation, triggering degradation of RPB1. **ARE-1** reduced nascent RNA in LREX' cells as measured by 5-EU incorporation (Fig. 3), and we have previously observed RPB1 degradation after long-term treatment with **ARE-1** and related polyamides (8,12). This suggests long-term treatment with **ARE-1** reduces global transcription in LREX' cells.

Suppression of enzalutamide resistant, castrate resistant CaP in vivo

We further tested the efficacy of **ARE-1** in VCaP xenografts, which exhibit modest response to 10mg/kg enzalutamide treatment, and in mice engrafted with enzalutamide resistant LREX' cells (5,13). In VCaP xenografts, **ARE-1** dose-dependently reduced tumor growth by 70% at 5mg/kg compared to vehicle (Fig. 4A) without significant toxicity (Supplementary Fig. S3A). In castrated mice bearing LREX' tumors, **ARE-1** and enzalutamide cotreatment reduced growth by 80% compared to enzalutamide alone (Fig. 4B) without significant toxicity (Supplementary Fig. S3B). Enzalutamide was administered daily post engraftment at 10mg/kg to maintain GR expression, which was confirmed by immunohistochemistry. LNCaP tumors, which do not express GR, were used as controls (Fig. 4C). Furthermore, LREX' tumors treated with **ARE-1** and enzalutamide showed reduced *KLK3* expression (Supplementary Fig. S3C), elevated TUNEL and reduced Ki67 staining compared to enzalutamide alone (Supplementary Fig. S3D). GSEA of tumor expression profiles show **ARE-1** treatment elicits similar UV response signatures as seen in cell culture, and represses ontologies associated with DNA binding dependent transcription (Supplementary Tables S2 and S3). Plasma concentration of **ARE-1** from terminal blood samples from LREX' engrafted animals was compared to the plasma concentration in C57BL6/J animals treated with 30mg/kg; AUC was 25.9 and 189.9 μ g*hr/mL, respectively (Supplementary Fig. S4). At 30mg/kg mice experienced a 6% weight loss but recovered within 5 days without visible signs of distress (not shown).

Discussion

AR LBD mutations, expression of transcriptionally active splice variants lacking the LBD, co-option of NHRs with similar DNA binding specificities, or loss of reliance on AR, may drive enzalutamide resistance (3). Furthermore, different metastatic foci within a patient may resist enzalutamide through different mechanisms (15), suggesting a successful treatment strategy might use multiple therapeutics that overcome different resistance mechanisms, or alternatively, a single therapeutic capable of overcoming multiple mechanisms. Therapeutic targeting of the NHR-DNA interface may overcome most known enzalutamide resistance mechanisms.

The GR antagonist mifepristone added to ADT was previously tested in mCRPC patients and was not effective(16). Trials for mCRPC patients combining enzalutamide with mifepristone are underway. Other NHRs may also be active in refractory CaP (3). Notably, progesterone receptor inhibitors have entered clinical trials for mCRPC. Therapeutics targeting the N-terminal domain (NTD) of AR, or that mediate degradation of AR, may overcome treatment resistance due to AR splice variants. The NTD inhibitor EPI-506 has entered clinical trials (17). However, this approach may not overcome resistance due to co-option of alternate NHRs. Others have reported small molecules that interfere with the AR DNA-binding domain (18). The clinical utility of this approach is unknown.

We report a Py-Im polyamide with activity against enzalutamide-resistant CaP in cell and animal models. Polyamide **ARE-1**, targeted to the sequence 5`-WGWWCW-3`, which is similar to the ARE and GRE half site, attenuates ligand induced AR and GR transcriptional activity, is more potent than enzalutamide and bicalutamide in cell culture, and is active against enzalutamide resistant xenografts. Long term treatment of LREX' cells with **ARE-1** also decreases nascent RNA synthesis. In biophysical experiments, polyamides can halt RNAP2 elongation directly upstream of a polyamide binding site (19). We hypothesize this stalling of RNAP2 promotes ubiquitination and degradation of RPB1, ultimately interfering with RNA synthesis, which may contribute to efficacy against treatment refractory CaP. Other molecules that interfere with RNA synthesis are proposed as potential drug candidates for CaP (13,20).

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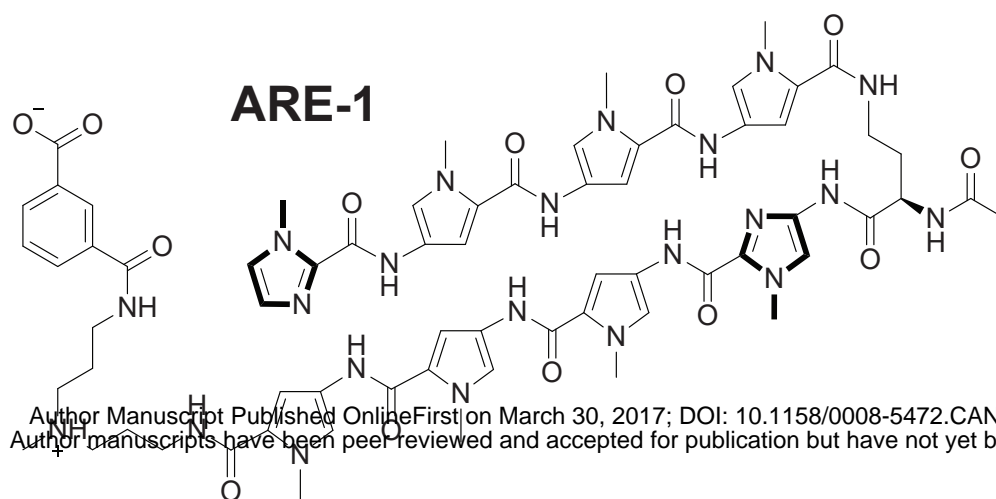
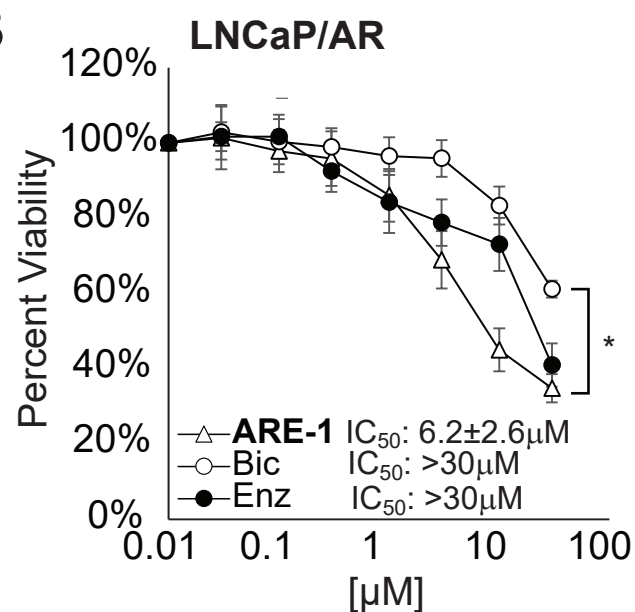
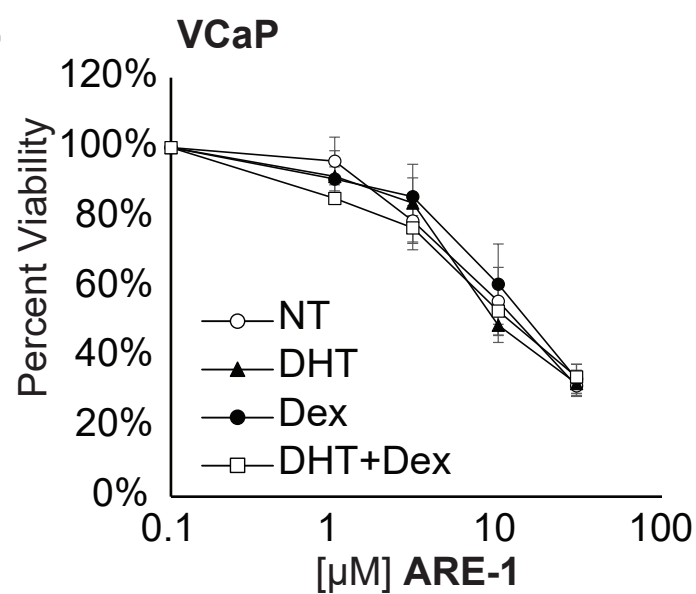
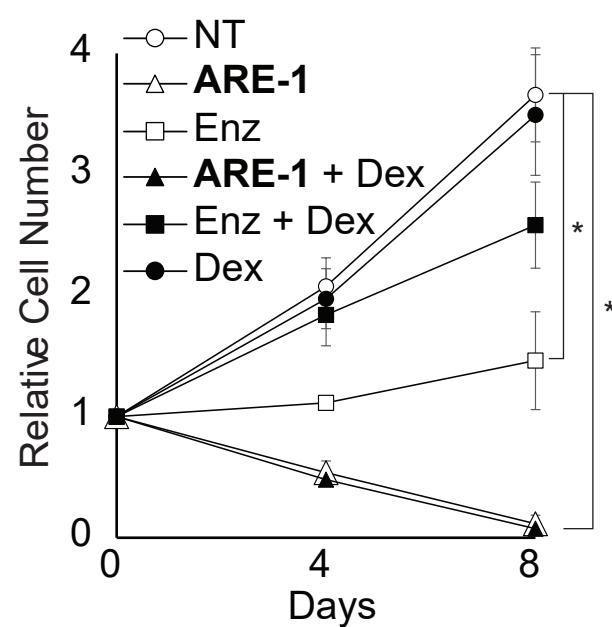
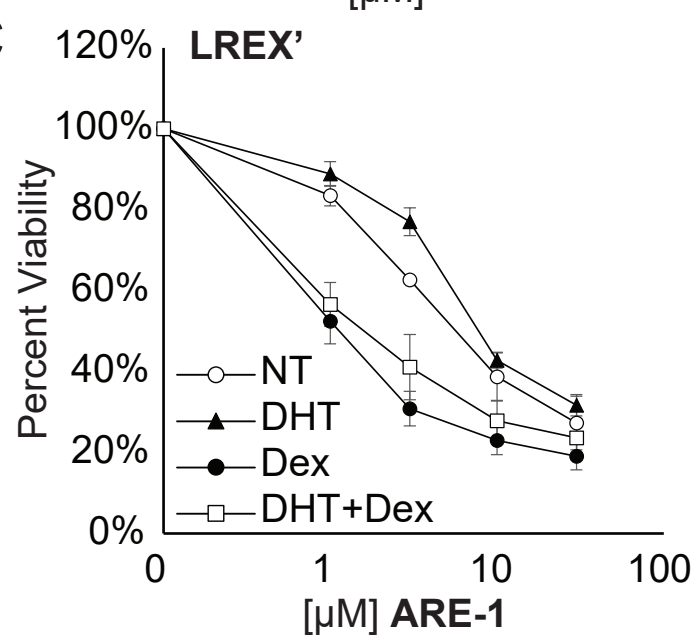
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Figure 1. A, structure of **ARE-1**. **B**, cytotoxicity of **ARE-1**, Bic, and Enz in LNCaP/AR cells over 72hours. **C**, cytotoxicity of **ARE-1** in LREX' cells is not attenuated by AR or GR activation. **D**, top, cytotoxicity of **ARE-1** in VCaP cells is not affected by Dex, bottom, long-term incubation of **ARE-1** or Enz in VCaP cells. Error bars are SEM. * $p < 0.05$.

Figure 2. A, effects of **ARE-1**, Bic, and Enz on DHT induced *KLK3* mRNA in LNCaP/AR cells (top), and baseline (charcoal stripped media) (bottom). **B**, effects of **ARE-1** and Enz against select genes co-regulated by AR and GR in LREX' cells. **C**, GSEA analysis in LREX' cells. Top, DHT enriches for the Androgen Response. Bottom, **ARE-1** with DHT negatively enriches for Androgen Response and the UV Response Down. **D**, **ARE-1** treatment of LNCaP cells negatively enriches for Androgen Response and the UV Response Down. * $p < 0.05$.

Figure 3. A, nascent RNA in LREX' cells treated with **ARE-1** for 48hours. Actinomycin D (Act D):positive control. **B**, nascent RNA in LREX' cells by flow cytometry after treatment with 10 μ M **ARE-1** for 24, 48, 72hours. **C**, composite of flow cytometry results. Error bars are SEM. * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$.

Figure 4. Mice were treated three times per week with **ARE-1** subcutaneously to flanks opposite engrafted tumor. **A**, left, tumor volumes and (right) final tumor masses of VCaP xenografts treated with vehicle (Veh)($n=8$), 2.5mg/kg($n=7$), and 5mg/kg($n=8$) **ARE-1**. **B**, left, tumor volumes and (right) final tumor masses of LREX' xenografts in castrated animals treated daily with 10mg/kg Enz and Veh($n=14$) or Enz and 2.5mg/kg **ARE-1**($n=12$). **C**, GR staining of LREX' and LNCaP tumors. All LREX' tumors stained for GR. Error bars for tumor volumes are SEM. Whisker plots represent means, standard deviations.

A**B****D****C****Figure 1.**

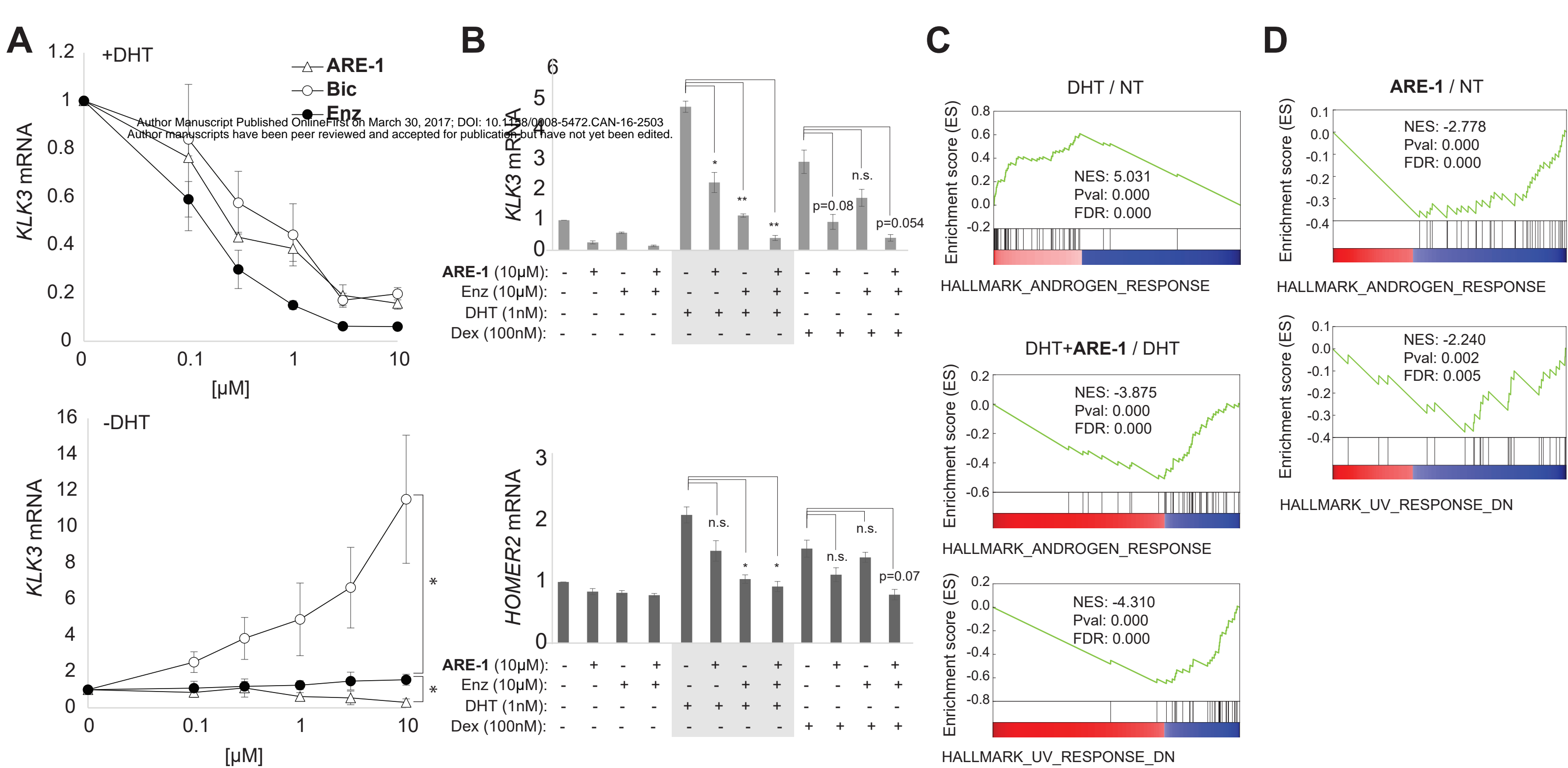


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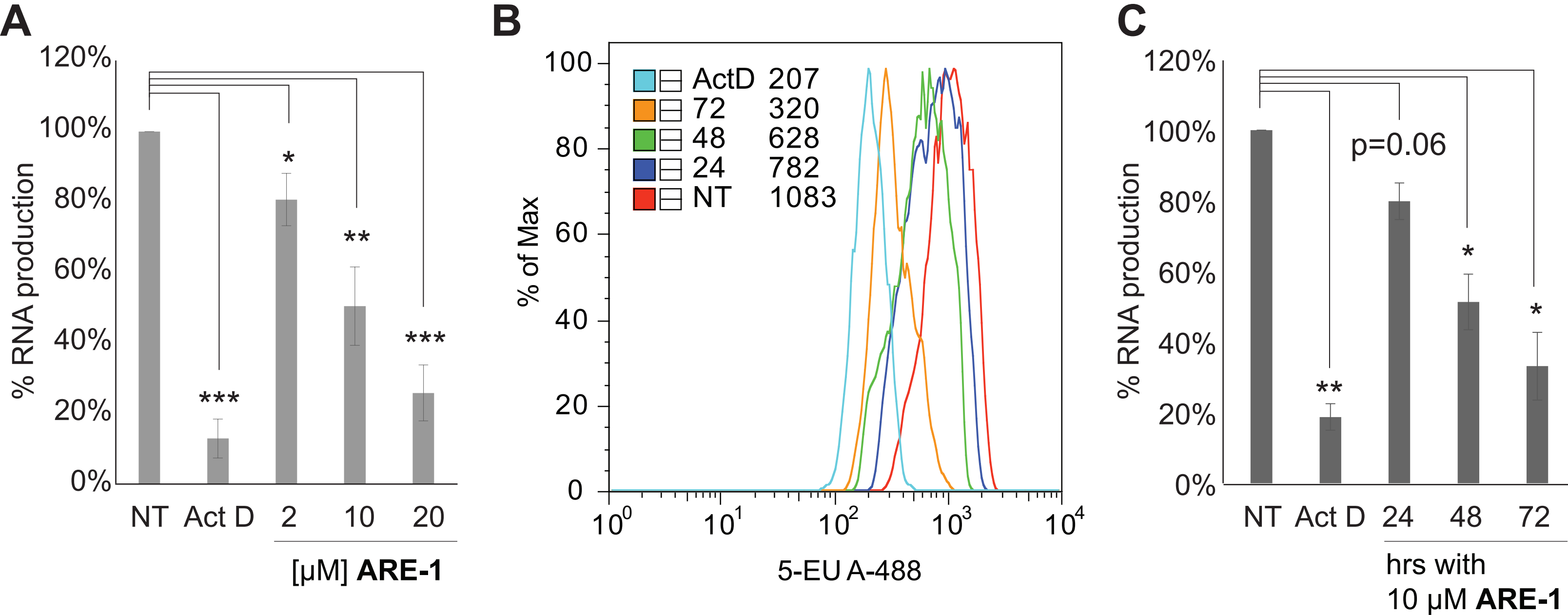


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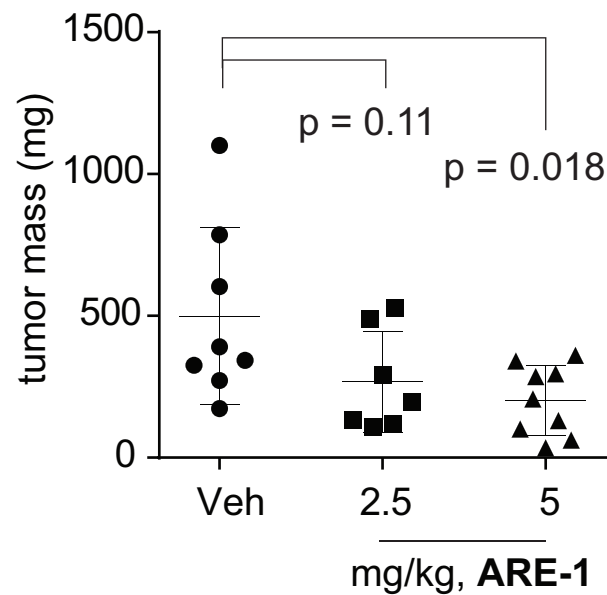
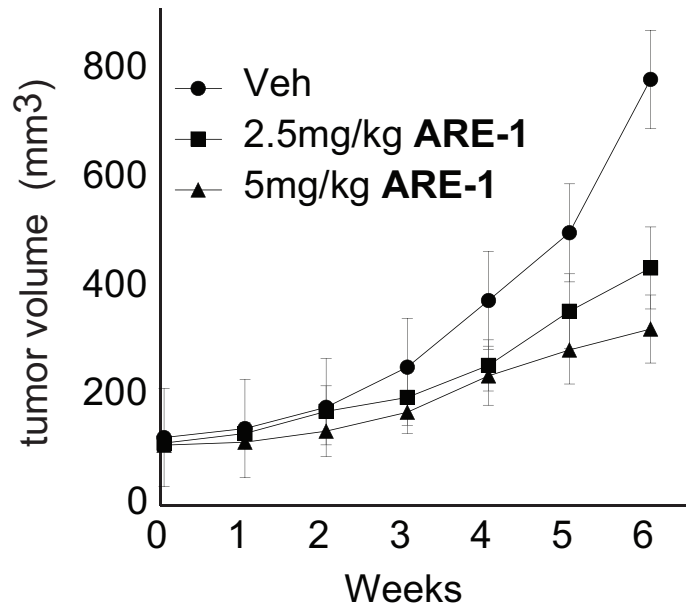
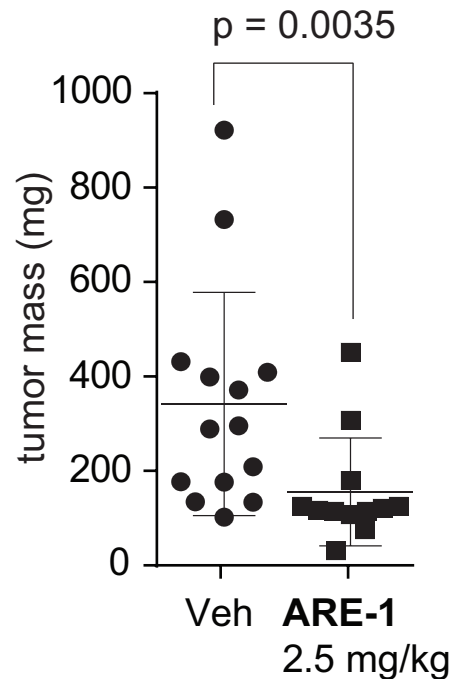
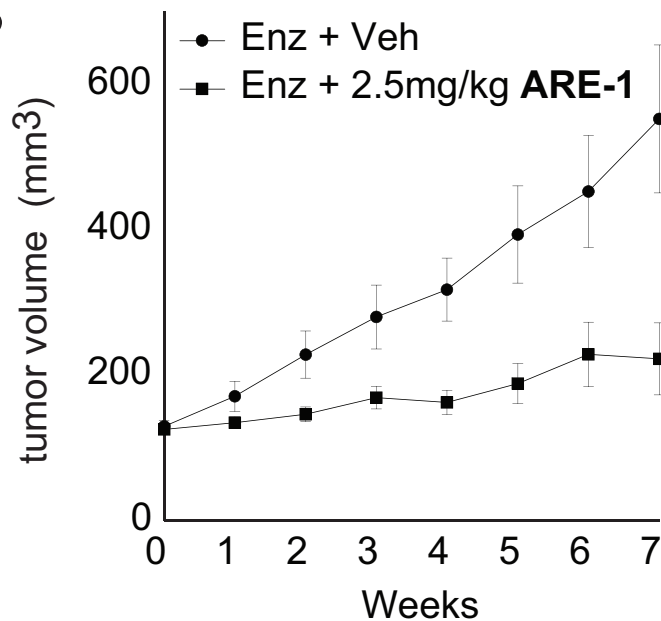
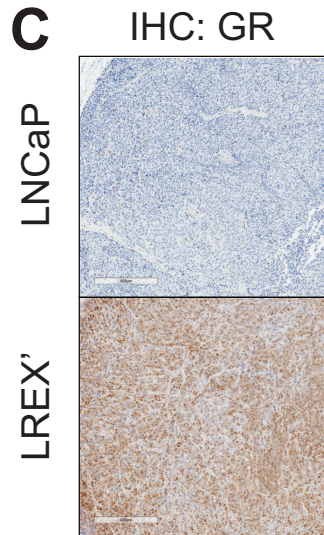
A**B****C**

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